

***Arabidopsis thaliana* as a model for the study of plant–virus co-evolution**

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Understanding plant–virus coevolution requires wild systems in which there is no human manipulation of either host or virus. To develop such a system, we analysed virus infection in six wild populations of *Arabidopsis thaliana* in Central Spain. The incidence of five virus species with different life-styles was monitored during four years, and this was analysed in relation to the demography of the host populations. Total virus incidence reached 70 per cent, which suggests a role of virus infection in the population structure and dynamics of the host, under the assumption of a host fitness cost caused by the infection. Maximum incidence occurred at early growth stages, and co-infection with different viruses was frequent, two factors often resulting in increased virulence. Experimental infections under controlled conditions with two isolates of the most prevalent viruses, cauliflower mosaic virus and cucumber mosaic virus, showed that there is genetic variation for virus accumulation, although this depended on the interaction between host and virus genotypes. Comparison of Q_{ST} -based genetic differentiations between both host populations with F_{ST} genetic differentiation based on putatively neutral markers suggests different selection dynamics for resistance against different virus species or genotypes. Together, these results are compatible with a hypothesis of plant–virus coevolution.

Keywords: host–pathogen coevolution; plant–virus coevolution; *Arabidopsis thaliana*; plant virus ecology; natural variation

1. INTRODUCTION

Pathogens are virulent parasites: infection results in damage to the host, which can be quantified as a reduction of the host fitness (e.g. Read 1994; Woolhouse *et al.* 2002). Hosts have developed different defence strategies to avoid or limit pathogen infection, or to compensate for its costs (Agnew *et al.* 2000). Among these, one major defence mechanism of plants against their pathogens is resistance, i.e. the ability of the host to limit parasite multiplication (Clarke 1986). As host defences may reduce the fitness of the parasite, hosts and parasites may co-evolve, defining coevolution as the process of reciprocally adaptive genetic change in two or more species (Woolhouse *et al.* 2002). It is currently assumed that plants and viruses co-evolve. However, this assumption is supported by surprisingly little evidence. Woolhouse *et al.* (2002) list three conditions that should be met

for host–pathogen coevolution: (i) genetic variation in the relevant host and pathogen traits (e.g. resistance, tolerance, infectivity, virulence); (ii) reciprocal effects of the relevant traits of the interaction on the fitness of host and pathogen; (iii) dependence of the outcome of the host–pathogen interaction on the combination of host and pathogen genotypes involved. For plants and viruses there is only partial evidence that these conditions are met. Evidence supports, mostly, the detrimental effects of highly virulent viruses in crop production, not necessarily linked to a fitness decrease, and the changes in the genetic structure of virus populations in agricultural ecosystems as a response to human manipulation of the genetics of the host by breeding resistance factors (Fraile & García-Arenal *in press*). Evidence for plant–virus coevolution would require the analysis of systems in which the host might evolve in response to virus infection, i.e. virus–host systems involving wild hosts in natural ecosystems. Currently, these analyses are lacking. Reports of negative effects of virus infection in wild plants in their natural habitats are scant (e.g. Maskell *et al.* 1999; Funayama *et al.* 2001; Power & Mitchell 2004; Malmstrom *et al.* 2005a,b, 2006) and indicate that effects may largely depend on site or host population (Pallett *et al.* 2002). It has been proposed even that,

in most cases, viruses would be mutualistic symbionts, rather than pathogens, in wild plant ecosystems (Roossinck 2005; Wren *et al.* 2006). To approach the analysis of plant–virus coevolution in wild ecosystems we have chosen *Arabidopsis thaliana* as a host plant.

Arabidopsis thaliana L. (Brassicaceae) is an annual species with a present worldwide distribution after expansion from its native geographical range in Eurasia and North Africa (Hoffmann 2002). Europe/North Africa has been proposed as a main centre of origin of *A. thaliana* (Hoffmann 2002). Within this region, the Iberian Peninsula is a centre of genetic diversity, and the genetic structure of *A. thaliana* in Iberia suggests that it harboured multiple glacial refugia during the Pleistocene, which contributed to the species; re-colonization of Europe in post-glacial times (Picó *et al.* 2008). In the Iberian Peninsula, *A. thaliana* can be found in a variety of anthropic and wild habitats (Picó *et al.* 2008) where plants flower mainly in spring. Demographical analyses show that populations are built of two or one cohorts of plants that either germinate in the autumn and overwinter as rosettes, or germinate in the spring. The occurrence and demographic relevance of each cohort depends on the site and the climatic conditions (Montesinos *et al.* 2009). For over 20 years, *A. thaliana* has been developed as the model organism for molecular plant genetics, including the analysis of the mechanisms of resistance to parasites (Sommerville & Koornneef 2002). More recently, *A. thaliana* has been increasingly developed as a model for plant ecology and evolutionary genetics (Mitchell-Olds & Schmitt 2006), including the study of the consequences of herbivory and parasitism on plant fitness, and plant–parasite coevolution (Kover & Schaal 2002; Salvaudon *et al.* 2005; Goss & Bergelson 2006). These analyses have not considered viral parasites, and there is currently no information on what viruses infect *A. thaliana* in nature or on the possible consequences of viral infection on the population dynamics and genetics of *A. thaliana*.

To address the role, if any, of virus infection on the evolutionary ecology of *A. thaliana*, we have monitored over four years the incidence of five virus species on six wild populations of *A. thaliana* in different habitats of Central Spain. The chosen viruses were cucumber mosaic virus (CMV), turnip mosaic virus (TuMV), turnip yellow mosaic virus (TYMV), turnip crinkle virus (TCV) and cauliflower mosaic virus (CaMV). These virus species were chosen based on different criteria: they have been shown to infect wild Brassicaceae species in their natural habitats in Europe (Maskell *et al.* 1999; Thurston *et al.* 2001; Pallett *et al.* 2002; Sacristán *et al.* 2004; Malpica *et al.* 2006). Also, they belong to different genera and families, and exemplify different genomic structures and genome expression strategies. Last, and perhaps most important, these five virus species exemplify different life histories, which could affect their ecology and evolution. CMV is a typical generalist, with the broadest host range for a plant virus, as it infects more than 1000 species in more than 100 families of monocotyledonous and dicotyledonous plants, TuMV has a moderate host range limited to few dicotyledonous families, while



Figure 1. Geographical location of *A. thaliana* populations in Central Spain. White dots indicate the site of the six populations surveyed in this work. Populations are numbered from North to South. The Sierra de Guadarrama mountains separate populations 1 and 2 from the rest.

TCV, TYMV and CaMV have natural host ranges restricted only to the Brassicaceae, with some additional host species of the related families Resedaceae and Caparidaceae for TYMV. CMV and TuMV are transmitted by aphids in a non-persistent manner, CaMV is aphid-transmitted in a bimodal manner, both non-persistently and semipersistently, whereas TCV and TYMV are transmitted by chrysomelid beetles. Last, only CMV is efficiently seed transmitted in different species of the Brassicaceae, including *A. thaliana* (Pagán & García-Arenal 2010, unpublished results). The molecular genetics of these viruses has been extensively analysed, and the population genetics of CMV and TuMV has also been studied in detail (for reviews, see Hollings & Stone 1972; Carrington *et al.* 1987, 1989; Haas *et al.* 2002; Walsh & Jenner 2002; Palukaitis & García-Arenal 2003).

The collected data on virus incidence and its relationship with some ecological parameters indicate that virus infection could affect the demography of *A. thaliana*. In addition, data on the genetic variation for resistance to the most prevalent viruses suggests that virus infection may have a role in shaping the genetic structure of *A. thaliana* populations. Together, these results are compatible with a hypothesis of plant–virus coevolution.

2. MATERIAL AND METHODS

(a) Field surveys

Six *A. thaliana* populations were studied in this work, whose location in Central Spain is shown in figure 1, and relevant ecological features are given in table 1. Except for the population at Las Rozas, located in an anthropic grassland, all other populations were located in more natural habitats, as indicated in table 1. In order to sample plants for virus detection, these populations were surveyed at two different dates during the spring of 2005, 2006 and 2007, when most plants were either at vegetative (rosette) or reproductive (flowering and fruit maturation) phases. At each survey, 5–45

Table 1. Geographical and ecological description of *A. thaliana* populations.

| site | altitude (m) | latitude/longitude | habitat | substrate |
|--------------------------|--------------|--------------------|--|----------------|
| Carbonero el Mayor (CEM) | 890 | 41°8' N/4°19' W | mediterranean evergreen <i>Quercus</i> forest | granite |
| Ciruelos de Coca (CDC) | 789 | 41°12' N/4°32' W | pine grove | siliceous sand |
| Las Rozas (LRO) | 724 | 40°30' N/3°53' W | mediterranean xeric grassland | siliceous sand |
| Polán (POL) | 513 | 39°49' N/4°15' W | mediterranean evergreen <i>Quercus</i> forest | granite |
| Menasalbas (MEN) | 735 | 39°39' N/4°20' W | wooded mediterranean xeric grassland | granite |
| Marjaliza (MAR) | 983 | 39°34' N/3°55' W | mediterranean evergreen <i>Quercus</i> shrubland | quartzite |

plants were randomly collected depending on population size, in at least three patches within the population. Collected plants were transplanted and kept in a growth chamber at 22°C, 16 h photoperiod, and two weeks later leaf tissues were harvested for virus detection. In 2008 a more detailed survey was carried out, in which populations were surveyed at five dates between late winter and late spring, from mid February to early June, and two times in the autumn, from early October to mid-November. At each survey, plant density was estimated by counting the number of *A. thaliana* plants in five 0.5 × 0.5 m quadrats 20 m distant from each other within fixed transects inside the population. The phenological stage of all the plants within the quadrat was recorded considering three categories: vegetative growth as a rosette (i.e. stages 1.04–3.9 of Boyes *et al.* 2001), reproductive growth with plants producing flowers and fruits (i.e. stages 5–6.90) and seed shattering (i.e. plants in which at least one silique had shattered, stages 8 and later). A variable number of randomly chosen plants within each quadrat (between 1 and 9 depending on the quadrat plant density) were collected for virus detection and handled as in previous years.

(b) Bioassays for virus resistance

Twenty randomly sampled individuals from Marjaliza (MAR) and Ciruelos de Coca (CDC) populations were assayed for their resistance/susceptibility to infection by CMV and CaMV under controlled conditions. These individuals were grown simultaneously in the same greenhouse to obtain by selfing the seeds used for the experiments described here. Offspring seeds of each individual were sown on filter paper soaked with water in single plastic Petri dishes, and stratified in darkness at 4°C for 3 days before transferring for germination to a growth chamber (22°C, 14 h light and 70% relative humidity). Five day-old seedlings were planted, one per pot, in soil containing pots 10.5 cm diameter and 0.43 l volume, and grown in a greenhouse (25°C/20°C day/night, 16 h light).

Plants were inoculated with two isolates of CMV and two isolates of CaMV: one well-characterized reference isolate of each virus (Fny-CMV and CaMV strain Cabb-S; Franck *et al.* 1980; Owen *et al.* 1990), and one isolate obtained from field-infected *A. thaliana* plants (At-CMV, isolated from a Ciruelos de Coca plant sampled in 2008, and At-CaMV, from Las Rozas, 2007). All viruses were multiplied in Chinese cabbage, and sap was obtained by grinding infected Chinese cabbage leaves in 0.01 M phosphate buffer pH 7.0, 0.2 per cent sodium

diethyldithiocarbamate was used to mechanically inoculate carborundum-dusted leaves of young *A. thaliana* plants. The (unknown) virus concentration in sap was sufficient to ensure infection of all inoculated plants. Inoculations were done at the vegetative growth stage, when plants had 4–5 rosette leaves. Each treatment (virus-inoculated or buffer mock-inoculated) involved five replicated sister plants from each original field individual. All plants of the 40 lines were simultaneously grown in the greenhouse in a completely randomized design. Fifteen days post-inoculation (dpi) 0.01 g (fresh weight) of leaf tissue was harvested from inoculated and systemically infected leaves for virus quantification.

These 40 individuals from MAR and CDC populations had been previously genotyped on the basis of 73 nuclear single nucleotide polymorphisms (SNPs), 16 nuclear microsatellites and four chloroplastic microsatellites (Picó *et al.* 2008).

(c) Virus detection and quantification

Infection by CMV, CaMV, TCV and TuMV in field plants was detected by hybridization of ³²P-UTP-labelled RNA probes, transcribed from cDNA clones, which were complementary to nucleotides 1861–2193 of Fny-CMV RNA3 (Acc. no. AF127 976), 1830–2219 of the genomic DNA of CaMV strain Cabb-S (Acc. no. NC001497), 37–181 of M-TCV genomic RNA (gRNA) (Acc. no. NC_003 821), and 9363–9827 of TuMV strain UK1 gRNA (Acc. no. AF169 561). Hybridizations were performed on impressions of leaf disks on nylon membranes. TYMV was detected by ELISA using commercial antisera (Sediag, Longvic, France).

Virus accumulation in plants of populations MAR and CDC infected under controlled conditions was quantified as viral RNA (RNA + DNA for CaMV) accumulation. Total nucleic acid extracts from leaf tissues were obtained using TRI-reagent (Sigma-Aldrich, St Louis, MO, USA). Viral nucleic acid quantification was done by dot-blot hybridization with the ³²P-labelled RNA probes specific for CMV and CaMV described above. In each blot, internal standards for Fny-CMV gRNA, or for CaMV Cabb-S DNA were included as a two-fold dilution series of purified nucleic acid (0.5–0.001 µg) in nucleic acid extracts from non-inoculated *A. thaliana* plants. Mock-inoculated samples were included as negative controls. Nucleic acid extracts from infected plants were blotted at different dilutions to ensure that hybridization signal was on the linear portion of the RNA (DNA) concentration–hybridization signal curve.

All hybridizations were done at 65°C overnight in 6× SSC, 5× Denhardt's mixture, 0.1 per cent sodium dodecyl sulphate and yeast tRNA at 50 µg ml⁻¹ (Sambrook & Russell 2001). RNA hybridization signal was detected using a Typhoon 9400 scanner (GE Healthcare, Chalfont St Giles, UK) after exposure of the Eu⁺² store phosphor screens to the labelled samples, and quantification was done with IMAGE-QUANT 5.2 (Molecular Dynamics, GE Healthcare). As loading controls, parallel membranes were hybridized with a cDNA probe of approximately 800 nucleotides long of barley 18S rRNA (Gerlach & Bedbrook 1979), which showed no significant difference in the amount of rRNA in extracts from equal fresh weights of leaves, among infected or mock-inoculated plants.

(d) Statistical analyses

Spatial distribution of plants within *A. thaliana* populations was analysed using the variance-to-mean ratio: $VM = s^2/x$, where s^2 is the variance, and x is the mean of the number of plants per quadrat. VM values lower than 1 are indicative of regular spatial distribution, equal to 1 of random distribution, and higher than 1 of aggregated distribution (Upton & Fingleton 1985). Significant departure from random spatial distribution was analysed using the transformation $C = (n - 1)VM$, n being the number of replicated quadrats, which has been shown to follow a χ^2 distribution (Upton & Fingleton 1985).

The variation for the year's maximum value of incidence (maximum incidence) of each virus across populations and years was analysed with generalized linear models (GLM) considering population site, virus species and year as random factors. Differences among population sites, virus species or year were analysed using least significant difference (LSD) and Student–Newman–Keuls post hoc tests. Since both test yielded similar results, only LSD analyses are shown. Data on maximum incidence was not homoscedastic and, therefore, incidence proportions were arcsin/√ transformed. Differences in this trait according to population site, virus species and year were also analysed using Kruskal–Wallis tests. Since GLM analyses led to the same conclusions as Kruskal–Wallis tests, for simplicity only GLM analyses are shown.

Data on the frequency distribution of virus infection in single- or mixed-infected plants were compared by contingency tables, from which the χ^2 statistic was calculated. The probability of rejecting the null hypothesis of equal distribution was derived from the simulation of 1000 tables in which marginal totals were fixed according to the data (Model III).

Correlation between plant density and maximum viral incidence was tested using Pearson coefficients. All statistical analyses were performed using the statistical software SPSS 13.0 (SPSS, Inc., Chicago, IL, USA).

Differences in viral accumulation according to population, host line, or virus isolate, were analysed by two way ANOVA using isolate and line, or population and isolate, as factors in a complete model. Broad sense heritabilities of virus accumulations were estimated for each population (MAR and CDC) and

each virus isolate, as $h^2 = V_G/(V_G + V_E)$. V_G and V_E are, respectively, the between family (group of five sister plants derived by selfing from each field individual) variance, and the residual variance, which were estimated by variance component analysis using the REML method included in SPSS 13 package. Between population genetic differentiation in quantitative traits was measured as Q_{ST} values, estimated as described by Le Corre (2005). Briefly, a nested analysis of variance was carried out using population and family (within populations) as random factors. Between populations (V_B) and average within population (V_W) were estimated by the REML method of variance component analysis, and Q_{ST} was calculated as $V_B/(V_B + V_W)$. Genetic differentiation for neutral markers was estimated as F_{ST} values that were calculated by analysis of molecular variance (AMOVA) using the program ARLEQUIN v3.1 (Excoffier *et al.* 2005). Analysis of molecular variance tests were performed using multilocus genotypes of microsatellites or SNPs previously obtained for CDC and MAR individuals (Pico *et al.* 2008). Both differentiation parameters (F_{ST} and Q_{ST}) measure the proportion of genetic variation that is present among populations in relation to the total amount of observed genetic variation (between- plus within-populations), and, therefore, they vary from values of 0 to 1.

3. RESULTS

(a) Environment and demography of *A. thaliana* wild populations

The six *A. thaliana* populations analysed were located in an approximately 300 km N–S transect in the Central Plateau of Spain, and were divided into a Northern and a Southern group by Sierra de Guadarrama (figure 1). All populations were located in acidic soils from different geological substrata, at altitudes between 500 and 900 m a.s.l. (table 1). The population at Las Rozas (LRO) was in an anthropic xeric grassland that has been pastured by sheep until recently, while the remaining populations were on more naturally disturbed habitats occupying openings among the major tree or shrub vegetation. Floristic formations differed largely among sites, although three of them (Marjaliza, MAR; Menasalbas, MEN; Carbonero el Mayor, CMA) were dominated by evergreen oak (*Quercus rotundifolia* Lam). Vegetation at Ciruelos de Coca (CDC) was dominated by *Pinus pinaster* Aiton and at Polán by *Q. rotundifolia* and *Q. coccifera* L. (table 1).

Surveys carried out during 2008 showed that the growth period of *A. thaliana* was different at the different sites, as indicated by the temporal variation of plant density and phenology (figure 2). At MAR and MEN, germination occurred in late March and most plants had completed their cycle by early June. In LRO and CDC, plants at the early vegetative stage were found early in February, indicating autumn or winter germination, and plants had completed their cycle in early June. In POL there was evidence of germination in autumn/winter as well as in early spring; plants that germinated early in spring had completed their cycle by early June, since no senescent plants

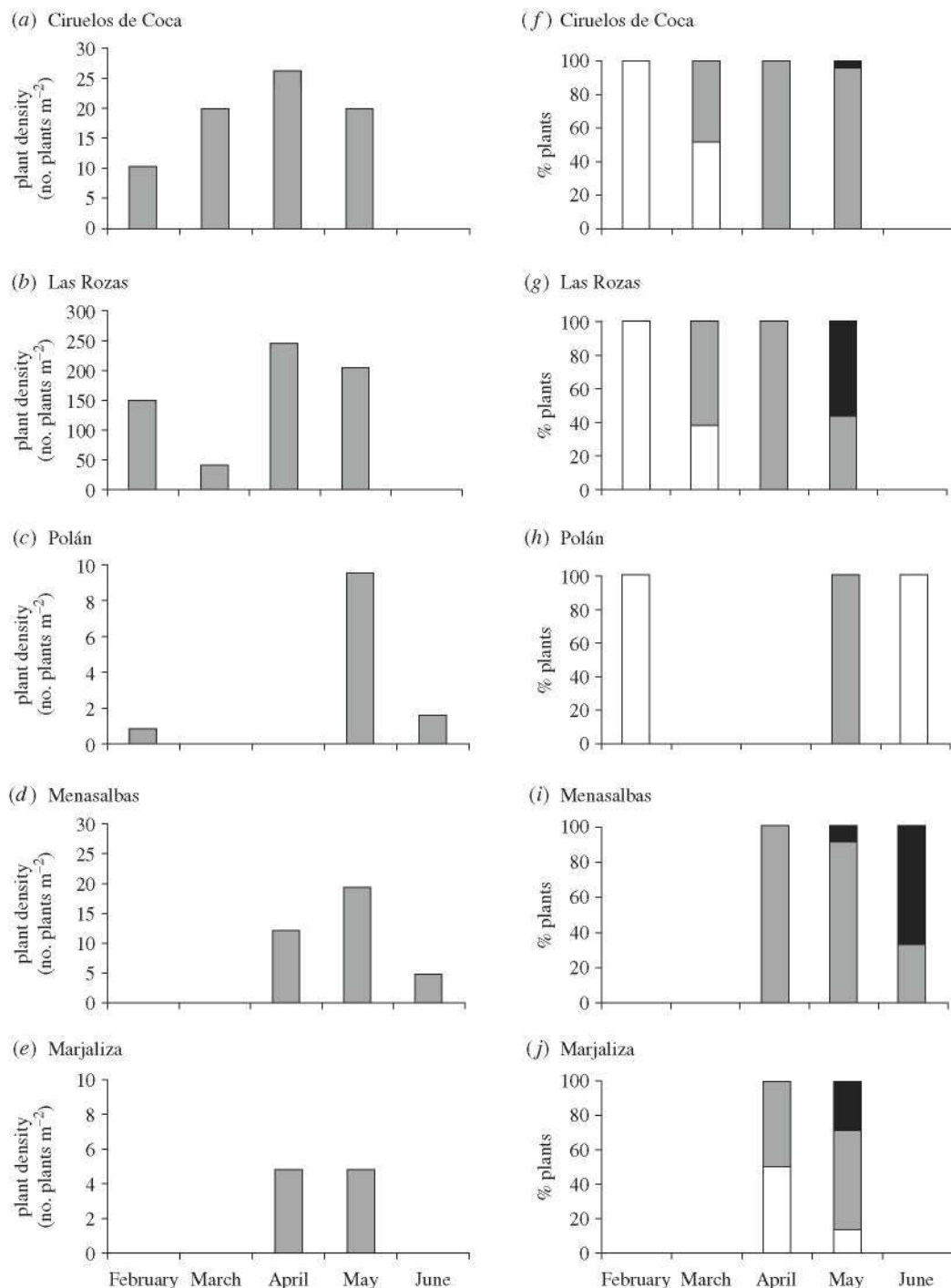


Figure 2. Temporal variation of plant density and phenological stage in *A. thaliana* populations in 2008. (a–e) show, for each *A. thaliana* population, the variation for plant density (measured as the number of plants per square metre) between February and June; (f–j) show the variation in phenology (measured as percentage of plants at each phenological stage). Three phenological stages are represented: vegetative growth as a rosette (phenological stages 1.04–3.90 of Boyes *et al.* (2001), white), reproductive growth with plants producing flowers and fruits (stages 5.10–6.90, grey) and seed shattering to senescence (plants in which at least one silique had shattered, stages 8.0–9.70, black).

were found at this last date. We did not find plants in any site in October, but in mid-November we found them at the early vegetative stage at MEN, POL, LRO and CDC, with densities varying between 0.8 plants m^{-2} (CDC) to eight or nine plants m^{-2} in MEN and LRO, respectively (data not shown). No plant was found at CMA at any date during 2008. Plant density varied significantly with population site, density at LRO being significantly higher than in the other sites ($p < 0.001$).

Plant counts in several 0.5×0.5 m quadrats in each population and at different survey dates were used for analysis of spatial distribution using the variance-to-mean ratio, VM (see §2). On average, plant distribution tended to be aggregated in the five sites, as shown by VM values, which were in most of the cases higher than 1 (mean values ranging between 0.4 and 393 depending on the population and survey date). However, significant departure from random spatial distribution (VM = 1), was consistently

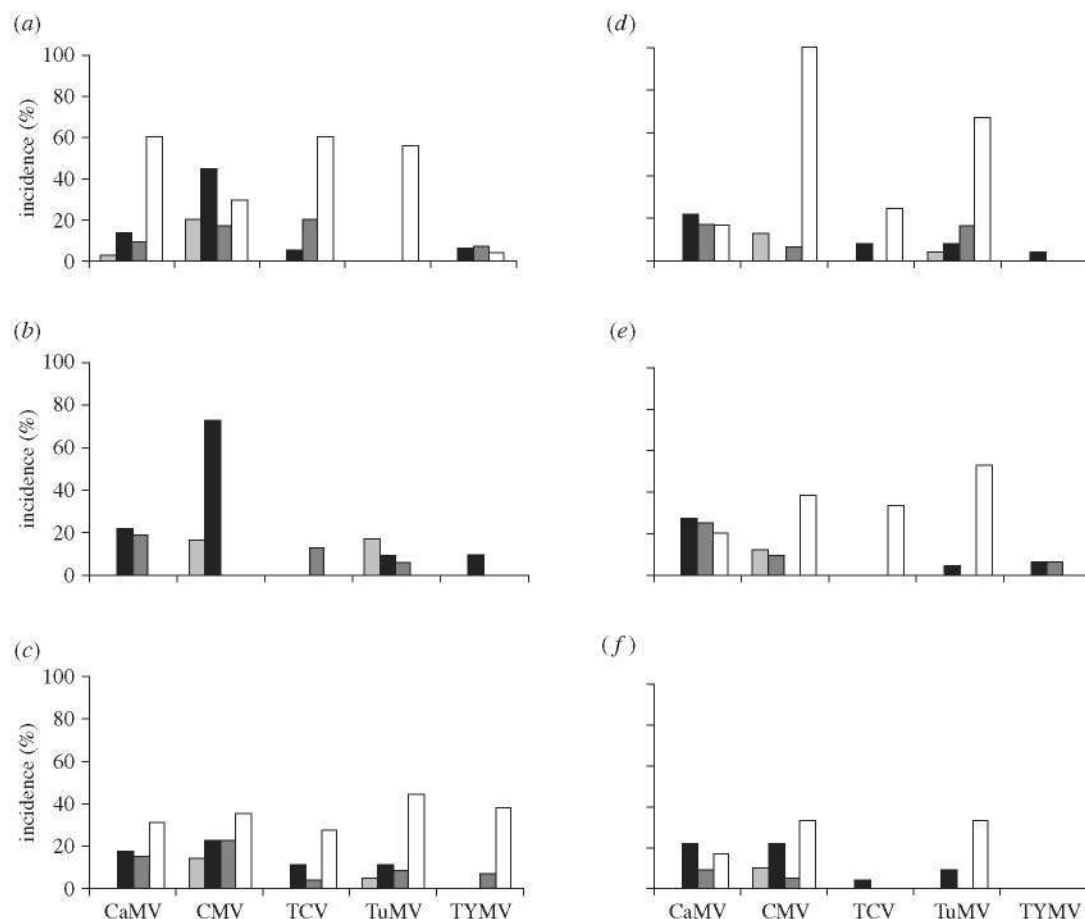


Figure 3. Annual viral incidence in *A. thaliana* populations. Incidences for five viruses (CaMV, cauliflower mosaic virus; CMV, cucumber mosaic virus; TCV, turnip crinkle virus; TuMV, turnip mosaic virus; TYMV, turnip yellow mosaic virus), recorded between 2005 and 2008 in the six *A. thaliana* populations ((a) Ciruelos de Coca; (b) Carbonero el Mayor, (c) Las Rozas, (d) Polan; (e) Menasalbas; (f) Marjaliza) monitored in this work. Incidence is shown as percentage of infected plants (light grey bar, 2005; black bar, 2006; dark grey bar, 2007; white bar, 2008).

observed only in LRO, and in the remaining populations only at dates with higher plant density (see the electronic supplementary material, table S1). Hence, these results indicate that aggregation, which is higher at higher plant density, is an intrinsic feature of the *A. thaliana* populations.

(b) Incidence of virus infection in wild *A. thaliana* populations

During the period 2005–2008, the five analysed virus species were detected every year in at least one *A. thaliana* population, except for TCV and TYMV, which were not detected in 2005 (see figure 3; the electronic supplementary material, table S1). Virus-infected plants did not show any specific symptom when collected in the field, or thereafter when transplanted and grown under controlled conditions. For each virus species, incidence varied largely according to host population site, year and date within a year (figure 3; the electronic supplementary material, table S1). The average maximum incidence of total viral infection over host populations was in 2008, with 63 per cent of infected plants, the maximum incidence in 2008 being observed in CDC (76%). Maximum incidence of total virus infection occurred

in Carbonero el Mayor (CMA) in 2006 and in CDC in 2008 (76%). The largest average maximum incidence for a single virus species over the six populations and four years was for CMV (24%). Incidence differed significantly according to virus species ($p = 0.021$) and according to year ($p = 0.003$), but not according to population site ($p = 0.259$), the interaction virus \times year and virus \times year \times site also being significant ($p < 0.043$). LSD analyses showed that virus incidence was different for each year ($p < 0.027$), which were graded as 2008 $>$ 2006 $>$ 2007 $>$ 2005. Incidence also differed for each virus species ($p < 0.024$), except for CaMV and TuMV, for which differences were marginally significant ($p = 0.054$), their incidences appearing in the order CMV $>$ CaMV \geq TuMV $>$ TCV $>$ TYMV (figure 3). A large fraction of infected plants was mixed-infected by two or more virus species. Of a total of 340 infected plants found over all sites and dates, 35.3 per cent were mixed-infected, but this fraction varied largely among sites and dates (between 20% and 100%, data not shown). Most plants infected by CaMV (58.2%), TYMV (64.7%), TuMV (69.2%) and TCV (76%) were co-infected by at least another virus species. For CMV, on the contrary, only 32.9 per cent of infected plants were co-infected with another virus.

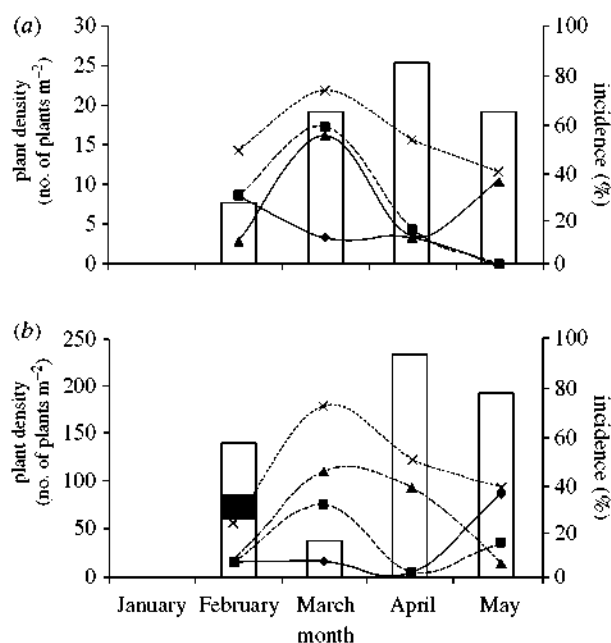


Figure 4. Comparison of the temporal variation in plant density and virus incidence in two *A. thaliana* populations ((a) Ciruelos de Coca, (b) Las Rozas) in 2008. Plant density and virus incidence were recorded between February and May. Plant density is measured as number of plants per square metre. Incidence of the three most prevalent viruses (CaMV, cauliflower mosaic virus, squares; CMV, cucumber mosaic virus, diamonds; TuMV, turnip mosaic virus, triangles) and total incidence of the five analysed viruses (crosses), are measured as percentage of infected plants.

Association analyses showed that CMV was found in co-infection with CaMV more than expected from random, CaMV significantly associated also with TuMV and TCV, and TuMV associated also with TCV ($p < 10^{-4}$). No significant association was found between TYMV and any other virus. The most common combinations of viruses occurring in co-infection were TuMV + TCV, CMV + CaMV and CaMV + TuMV + TCV (25.0%, 13.3% and 10.1% of mixed-infected plants). Sixty-two per cent of mixed-infected plants (21.8% of all infected plants) were infected by the potyvirus TuMV.

In 2008 an analysis of the temporal variation of virus incidence in relation to the demography of the host was undertaken. As shown in figure 4, considerable variation was found over the year for the incidence of virus infection at CDC and LRO populations, for which more data are available. In spite of habitat differences, the fraction of virus-infected plants showed a similar pattern of variation in both sites, with a maximum at early spring. Interestingly, both populations showed substantial virus incidence in late winter (23% at LRO, 50% at CDC), when plants were at the vegetative stage, and reached its maximum in early spring (more than 70% of infected plants in both populations), when the transition to reproductive growth was occurring (compare figures 2 and 4). The pattern of variation of the incidence of the three more prevalent viruses, CMV, CaMV and TuMV, was significantly different. The dynamics of CaMV and TuMV were similar for both populations,

with a maximum in early spring. However, CMV showed different dynamics to CaMV and TuMV for both populations: in CDC, CMV incidence was highest late in the winter, suggesting infection occurred during the autumn or early winter, while in LRO CMV incidence was highest at the end of spring. In plants sampled in November, infection by CMV and TCV was detected in MEN, POL and CDC; TuMV was detected in MEN and POL, whereas CaMV and TYMV were not detected in any population. Total virus incidence in November was 30 per cent in MEN and 50 per cent in POL; in CDC only one plant, virus-infected, was found.

It has been hypothesized that incidence of plant pathogens is favoured by high plant density. Considering the 2008 data for the five host populations, virus incidence was unrelated to plant population size ($p > 0.377$ for any virus species, $p = 0.090$ for total virus infection), suggesting that incidence depends on conditions external to the host population. For LRO and CDC populations, the relationships between virus incidence and plant density was also analysed by comparing both variables in the different quadrats sampled within each population at the different times of the year. Pearson's correlation analyses showed no correlation between plant density and virus incidence for any of the virus species or for total virus infection, at any date ($r < 0.76$, $p > 0.13$).

(c) Analyses of genetic variation for resistance/susceptibility to virus infection

Twenty randomly chosen individuals from the Ciruelos de Coca and Marjaliza populations were tested for their susceptibility to two CMV and two CaMV isolates, estimated as virus accumulation (table 2). One isolate of each virus was obtained from two of the *A. thaliana* Iberian populations analysed in this work (At-CMV and At-CaMV), while the other two isolates are well known reference strains (CMV-Fny and CaMV Cabb-S; see §2). All assayed individuals were systemically infected by the four virus isolates, and no immunity or hypersensitive resistance (HR) reaction was detected. Large amounts of genetic variation were found for the accumulations of the four isolates within one or both populations (table 2). Virus accumulation varied significantly with host genotype ($p < 0.0001$), heritabilities varying from 0.05 to 0.77 depending on isolate and population.

In the CDC population, susceptibility to both CMV genotypes did not differ significantly ($p = 0.08$), virus accumulation averaging 0.68 ± 0.64 and $0.42 \pm 0.62 \mu\text{g viral RNA g}^{-1}$ fresh leaf. In contrast, virus accumulation of the two CMV isolates was significantly different in the MAR population, which showed higher average susceptibility to Fny-CMV than to At-CMV (average accumulations of 7.39 ± 5.44 and $0.27 \pm 0.30 \mu\text{g viral RNA g}^{-1}$ fresh leaf, respectively, $p < 0.0001$; table 2). Thus, in MAR, virus accumulation depended on the virus genotype \times host genotype interaction ($p < 0.0001$). Accordingly, low genetic differentiation between both populations was found for At-CMV accumulation (Q_{ST} value of

Table 2. Variation for virus accumulation among *Arabidopsis thaliana* plants of Ciruelos de Coca (CDC) and Marjaliza (MAR) populations^a

| virus isolate | parameter | <i>A. thaliana</i> population | |
|---------------|----------------------------|-------------------------------|------------------|
| | | CDC | MAR |
| Fny-CMV | population mean \pm s.d. | 0.68 \pm 0.64 | 7.39 \pm 5.44 |
| | min–max genotype | 0.02–2.57 | 0.35–19.17 |
| | h_b | 0.05 | 0.62 |
| At-CMV | population mean \pm s.d. | 0.42 \pm 0.62 | 0.27 \pm 0.30 |
| | min–max genotype | 0.01–2.42 | 0.01–1.12 |
| | h_b | 0.38 | 0.10 |
| CaMV Cabb-S | population mean \pm s.d. | 15.15 \pm 10.90 | 10.71 \pm 5.59 |
| | min–max genotype | 2.04–30.76 | 2.78–21.56 |
| | h_b | 0.71 | 0.28 |
| At-CaMV | population mean \pm s.d. | 17.56 \pm 11.20 | 10.62 \pm 7.17 |
| | min–max genotype | 1.29–35.15 | 1.23–24.80 |
| | h_b | 0.77 | 0.54 |

^a For each virus isolate, accumulation is expressed as μg viral RNA g^{-1} fresh leaf.

h_b = Broad-sense heritability.

about 0), while a high differentiation was found for Fny-CMV accumulation (Q_{ST} value of 0.63).

For both *A. thaliana* populations, the accumulation of the two CaMV isolates was similar ($p = 0.47$), but was significantly higher ($p < 0.0001$) for CDC than for MAR. In CDC virus accumulation depended on the interaction virus genotype \times host genotype ($p = 0.002$; table 2). Genetic differentiation between these populations was rather low for the susceptibility to both CaMV isolates (Q_{ST} values of 0.10 and 0.20 for CaMV Cabb-S and At-CaMV, respectively).

4. DISCUSSION

The study of plant–virus coevolution requires wild systems in which, at odds with agricultural ones, there is no human manipulation of either the host or the pathogen. Hence, information on which viruses infect wild plants, the significance of virus incidence, and the possible effects of infection on plant fitness, should be acquired previous to the analysis of plant–virus coevolution. Information on these topics is presently very limited (Cooper & Jones 2006). Inventories of viruses infecting wild plants in pristine habitats have been undertaken only recently. Results indicate high prevalence of mostly non-described virus species, and suggest that these viruses could be mutualistic symbionts rather than pathogens (Roossinck 2005; Wren *et al.* 2006; Melcher *et al.* 2008; Muthukumar *et al.* 2009). Most analyses of virus infection on wild plant populations have focused on economically important virus species, and on the possible role of wild plants as inoculum reservoirs for epidemics on crops or as sources of new virus genotypes (e.g. Duffus 1971; Rist & Lorbeer 1991; Hobbs *et al.* 2000; Sacristán *et al.* 2004; Tugume *et al.* 2008). In some instances, it has been shown that these ‘crop’ viruses had a negative effect on their wild host that could affect the dynamics and structure of the wild population (Yahara & Oyama 1993; Maskell *et al.* 1999; Funayama *et al.* 2001; Power & Mitchell 2004; Malmstrom *et al.* 2005a,b, 2006). It is

usually unknown if the wild host was colonized by viruses from crops, or the other way around, but examples of both situations have been documented (Jones 2009). Thus, it is often unknown for how long viruses have interacted with their wild host, potentially leading to plant–virus coevolution. It has been suggested that the expansion of agriculture resulted in an increase and diversification of plant virus populations (Fargette *et al.* 2008; Gibbs *et al.* 2008), which might have led to spill-over to wild hosts. Under this scenario, the encounter of some crop viruses and the wild host might not have occurred earlier than 8000 years ago in regions such as the Iberian Peninsula (Pinhasi *et al.* 2005; Balaesque *et al.* 2010).

According to the above considerations, we analysed the extent of infection by some well-characterized viruses in wild populations of *A. thaliana*, to explore the adequacy of this plant as a model for studying plant–virus coevolution. We focused on five virus species that were selected by having different life histories, and that had been previously reported infecting wild species of the Brassicaceae in Europe (Pallett *et al.* 2002; Sánchez *et al.* 2007) and/or in Central Spain (Sacristán *et al.* 2004; Malpica *et al.* 2006; Pagán & García-Arenal 2010, unpublished results). We chose six *A. thaliana* populations located in different habitats. The factor habitat may determine the population dynamics of the analysed viruses and their vectors, as well as the demography of the host plant and, thus, the opportunity of infection. Four of the five virus species (all but TYMV) were found infecting the six analysed *A. thaliana* populations, but CMV and CaMV were most consistently detected over the years for all six sites. Interestingly, and despite important differences among sites in habitat and in the demography of the host plant, virus incidence did not differ among sites during the analysed period. Since populations at the different sites differed largely in density, this finding does not support the hypothesis that increasing host density will favour pathogen infection by facilitating encounters between infected and uninfected host (Burdon & Chilvers 1982). In

addition, no correlation was found between virus incidence and density among patches. Therefore, the aggregate ('patchy') distribution of plants within each population, with aggregation increasing with plant density, seems not to condition the possibility of virus transmission and, consequently, incidence. A possible explanation for this finding might be that the presence of other hosts for the analysed viruses and their vectors would contribute to the total density of susceptible host plants between patches and populations, and this total host density might determine the infection. On the other hand, as it has been often reported for crops (see Luis-Arteaga *et al.* 1998; Alonso-Prados *et al.* 2003; Moreno *et al.* 2004 for specific references of this same region), virus incidence varied largely from year to year, which has been related to the effect of climatic conditions on vector populations (Alonso-Prados *et al.* 2003). On the other hand, mixed-infections by two or more viruses were frequent. Interestingly, the most frequent associations could not be explained by shared vectors of the involved virus species or by a common mechanism of transmission, in agreement with other reports (Malpica *et al.* 2006). Thus, it could be speculated that infection by one virus makes the plant more susceptible to other viruses or more attractive to the vector(s) of a second virus (Mauck *et al.* 2010).

Several results on the incidence of virus infection suggest a possible role of these viruses in shaping the population structure and dynamics of *A. thaliana* in Central Spain. First, incidences reach rather high values, with up to 70 per cent of plants infected, and therefore it can be hypothesized that if virus infection had a negative effect on host fitness, it could be an important selective force on *A. thaliana* populations. Second, incidence was maximal when most, or a large fraction of, plants were at the vegetative stage. It is known that the negative effect of virus infection on growth and reproduction of many host plants, including *A. thaliana*, is often higher with earlier infection relative to the plant's life cycle (Matthews 1991; Pagán *et al.* 2007). Last, a large fraction of infected plants were mixed-infected by two or more virus species, and a large fraction of mixed infections involved the potyvirus TuMV. Mixed infections between taxonomically distinct plant viruses often result in synergism, i.e. increased multiplication of at least one partner and increased virulence (Roossinck 2005). Although no synergism has been reported for the interaction of CaMV and TuMV in *A. thaliana* (Martín & Elena 2009), most reported examples of synergism in different hosts involve a potyvirus and another unrelated species (Vance 1991; Wang *et al.* 2002; Murphy & Bowen 2006), and synergism may be strain-specific (Hii *et al.* 2002). The impact of CMV infection, the most prevalent of the analysed viruses, on the fitness of *A. thaliana* has been previously analysed experimentally. Thus, Pagán *et al.* (2007, 2008) characterized the response of 18 *A. thaliana* wild genotypes to infection by three CMV genotypes. CMV infection negatively affected plant fitness, estimated as viable seed production, regardless of symptom expression. Virulence depended on the specific virus genotype \times host genotype interaction,

but it was always higher when the plants were infected at the vegetative stage than at the reproductive stage. *A. thaliana* genotypes differed largely in their resistance and tolerance to CMV. Moreover, it was shown that both CMV virulence and plant defence were modulated by plant density, in a complex interplay of the direct and indirect costs of virus infection (Pagán *et al.* 2009). This series of reports, together with the field data presented here, strongly suggest that, at least for CMV, virus infection could be a selection force acting on the analysed *A. thaliana* populations. However, the intensity of such a force will depend on the interaction among several factors, including the genetic structure of both the virus and host populations, the time of infection relative to plant development, the effect of plant density on competition with uninfected plants of *A. thaliana* and/or other species, and the direct and indirect costs of infection for each specific genotype \times genotype interaction. Hence, it is currently not possible with the available data to determine the role of virus infection in the evolution of wild *A. thaliana*.

On the other hand, we have also analysed if there is genetic variation for the susceptibility to virus infection within and between wild *A. thaliana* populations, and if the accumulation of the virus depended on virus genotype \times host genotype interaction. For this analysis we chose the two most prevalent viruses, CMV and CaMV, and the two most geographically distant *A. thaliana* populations, CDC and MAR. For each virus, two genotypes were assayed, and for each *A. thaliana* population, 20 randomly sampled individuals were assayed. Results showed evidence of considerable genetic variation for the susceptibility to both viruses in both host populations, broad sense heritabilities of this trait over all families ranging from moderate (0.30 for At-CMV accumulation) to high (around 0.70 for Fny-CMV and At-CaMV). In addition, virus accumulation, a relevant trait of the plant-virus interaction, depended on the specific virus genotype \times host genotype interaction. These results parallel those reported since more than two decades ago for cellular plant pathogens (e.g. Parker 1985; Alexander 1989; Bevant *et al.* 1993), indicating polymorphisms for resistance in the host population. Furthermore, these relevant traits of the interaction depended on the specific host genotype \times pathogen genotype interaction, which have been interpreted as supporting the hypothesis of host-pathogen coevolution (Kaltz & Shykoff 1998; Salvaudon *et al.* 2008). Analyses of a variety of wild host species, including *A. thaliana*, and a variety of pathogens, including fungi, oomycetes and bacteria, have been reported (e.g. Kaltz *et al.* 1999; Kaltz & Shykoff 2002; Pei *et al.* 2002; Thrall *et al.* 2002; Laine 2005; Salvaudon *et al.* 2005; Goss & Bergelson 2006; Sicard *et al.* 2007), but we are not aware of similar studies with plant viruses. Genetic variation for resistance to viruses is well known to occur among genotypes of wild relatives of crops, which are important resources for breeding resistance to viruses into crop cultivars. Most data on virus resistance in wild crop relatives refer to qualitative resistance determined by either dominant or recessive major genes (Kang *et al.* 2005; Maule *et al.* 2007). Dominant monogenic HR to

CMV has been described in some *A. thaliana* ecotypes, and is determined by an allele (*RCY-1*) at a locus determining resistance to TCV and to the oomycete *Hyaloperonospora parasitica* (Takahashi *et al.* 1994, 2002). This resistance allele seems to be uncommon among *A. thaliana* genotypes, as it was detected in three out of 183 assayed ecotypes (Takahashi *et al.* 2002). We did not find HR reactions to CMV or CaMV in our assays, but resistance was quantitative and expressed as restricted virus accumulation. Although quantitative resistance of plants to pathogens has received less attention than dominant or recessive qualitative resistance, it has been proposed that it might play an important role in host defence and in host–pathogen coevolution (Salvaudon *et al.* 2008).

The results of the present study show that two of the three conditions pointed out by Woolhouse *et al.* (2002) as required for hosts and pathogens to co-evolve, are met in the analysed system: relevant traits of the interaction depend on the interacting host and pathogen genotypes, and there is genetic variation in the host and the pathogen for these traits. Also, host resistance will result in a diminished fitness of the pathogen, as resistance reduces the within-host component of pathogen fitness as well as the between-host component, since for aphid-transmitted viruses it has been repeatedly shown that efficiency of between-host transmission is positively correlated with multiplication in the plant that is the inoculum source (see Fraile & García-Arenal *in press*). It is presently unknown, however, if virus infection reduces the fitness of *A. thaliana* plants in the analysed populations. Comparisons of genetic differentiation among populations for neutral markers (F_{ST} estimates) with that observed for quantitative traits (analogous Q_{ST} estimates) provide an approach to determine the relative contribution of genetic drift and natural selection in explaining among-population divergence (reviewed by Leinonen *et al.* 2008). The individuals from CDC and MAR populations assayed here for susceptibility to CMV and CaMV have been previously genotyped using presumably neutral markers (Picó *et al.* 2008). From this, it has been estimated that the F_{ST} genetic differentiation between CDC and MAR populations varies from 0.29 for microsatellites to 0.48 for SNP markers (Picó *et al.* 2008). Q_{ST} -based genetic differentiations between both populations for the accumulation of the two CaMV isolates and of At-CMV were smaller than the above F_{ST} values estimated for neutral markers. These results suggest convergent selection in these two *A. thaliana* populations for resistance to those three virus genotypes. In contrast, the Q_{ST} differentiation for the accumulation of Fny-CMV was higher than F_{ST} values, suggesting divergent selection in both host populations for resistance to a foreign virus genotype, which has been shown to accumulate to high levels and to be highly virulent in most assayed hosts (Roossinck & Palukaitis 1991). Together, these analyses suggests different selection dynamics for resistance against different virus species and/or genotypes, which would be compatible with a differential negative effect of virus infection on *A. thaliana* and,

again, with the hypothesis of plant–virus coevolution. Further Q_{ST}/F_{ST} analyses including larger numbers of populations from a broader environmental diversity, will provide deeper insight into the evolutionary forces that drive the genetic differentiation of wild *A. thaliana* and virus populations.

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